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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> TARGETING OF LIPOSOMES TO THE BLOOD-BRAIN BARRIER  <b>(57) Abstract</b>  The present invention is directed to the targeting of drugs, peptides, and proteins to the brain. Liposomes containing the compound to be delivered are coupled to antibody binding fragments which bind to receptors on the vascular endothelium of the blood-brain barrier. The targeted receptors include transferrin receptor, insulin receptor, IGF-I and IGF-II receptors, among others. Also provided are pharmaceutical compositions and methods for administering the drugs to a subject in need by means of the receptor-targeted immunoliposomes.		

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## TARGETING OF LIPOSOMES TO THE BLOOD-BRAIN BARRIER

10

Background of the Invention

This invention relates generally to the delivery of drugs into the brain by transcytosis across the blood-brain barrier. More particularly, the invention relates to the targeting of drug-containing liposomes to the blood-brain barrier by means of antibodies and binding fragments thereof which bind to receptors on capillary endothelial cells of the brain.

Delivery of many drugs, particularly nonlipophilic drugs, to the mammalian brain is restricted by the blood-brain barrier. The barrier is due in part to tight intercellular junctions between brain capillary endothelial cells and prevents the passive movement of many substances from the blood to the brain. The brain endothelial cells lack continuous gaps or channels connecting the luminal and abluminal membranes which would otherwise allow the passage of blood-borne molecules into the brain tissue. Instead, the presence of specific transport systems within the capillary endothelial cells, such as those for insulin, amino acids, glucose and transferrin, assure the controlled transport of compounds necessary to the functioning of the brain.

Various strategies have been developed to deliver drugs into the brain that would not otherwise be able to cross the blood-brain barrier. Commonly, although quite undesirably, an intraventricular catheter is surgically implanted to deliver a drug directly into the brain. Not only does this involve an invasive procedure that itself is potentially harmful to the patient, but the drug delivered by

this means is only superficially distributed within the brain.

Other strategies have been devised to circumvent the blood-brain barrier. Some pharmacologic approaches involve chemically converting hydrophilic drugs into more lipid-soluble forms so they are more easily transported across the barrier. Another approach involves transiently opening the barrier by infusing hypertonic substances intra-arterially to allow passage of hydrophilic drugs, although hypertonic substances may be associated with toxicity and even damage the barrier.

More recently, it has been proposed to link hydrophilic neuropharmaceutical agents such as neuropeptides to other peptides which are themselves capable of crossing the blood-brain barrier by transcytosis. U.S. Patent No. 4,801,575 to Pardridge describes the preparation of chimeric peptides by coupling or conjugating the pharmaceutical agent to a transportable peptide. The chimeric peptide purportedly passes across the barrier via receptors for the transportable peptide. Transportable peptides, or vectors, mentioned as suitable for coupling to the pharmaceutical agent include insulin, transferrin, insulin-like growth factors I and II, basic albumin and prolactin. However, as noted in Pardridge et al., J. Pharm. Exp. Therap. 259:66-70 (1991), the use of insulin as a vector to the blood-brain barrier may be limited by hypoglycemia side effects and by rapid clearance of the peptide by the liver, and transferrin may be limited as a vector by its high concentration in the plasma. Friden et al., Proc. Natl. Acad. Sci. USA 88:4771-4775 (1991), have suggested that drugs conjugated to anti-transferrin receptor antibodies cross the blood-brain barrier.

It has also been suggested that liposomes can enhance drug delivery to the brain across the blood-brain barrier. See, e.g., Umezawa and Eto, Biochem. Biophys. Res. Comm. 153:1038-1044 (1988); Chan et al., Ann. Neurol. 21:540-547 (1987); Laham et al., Life Sciences 40:2011-2016 (1987); and Yagi et al., J. Appl. Biochem. 4:121-125 (1982). Liposomes are small vesicles (usually submicron-sized) comprised of one or more concentric bilayers of phospholipids

s parat d by aqueous compartments. Although liposom s hav  
be n report d to nhance th uptak of certain drugs into the  
brain after intravenous injecti n (Chan t al., and Laham t  
al., ibid.), it has more recently been shown that liposomes do  
5 not cross the blood-brain barrier. Schackert et al., Select.  
Cancer Therapeut. 5:73-79 (1989) and Micklus et al., Biochim.  
Biophys. Acta 1124:7-12 (1992). As noted in Micklus et al.,  
ibid., liposomes circulating in the plasma are ultimately  
10 taken up by the liver, digested and the lipid components  
released and redistributed to other organs. See also, Derksen  
et al., Biochim. Biophys. Acta 971:127-136 (1988). The  
radioactivity found in the brain following intravenous  
injection of labeled liposomes of Umezawa and Eto, supra, and  
others may in fact be derived from digested lipids and not  
15 from the intact liposomes themselves.

Many drugs, particularly neuropeptides, growth  
factors and the like, are rapidly degraded in the systemic  
circulation prior to reaching the brain, or minimally  
penetrate the blood-brain barrier. Many of these peptides, if  
20 effectively delivered to the brain in small quantities, could  
have a dramatic therapeutic effect. See, e.g., Banks and  
Kastin, Am. J. Physiol. 259:E1-E10 (1990). Presently,  
however, large amounts must be administered to allow entry of  
even minimal levels required for pharmacologic action.

25 What is needed in the art is an effective means to  
deliver pharmaceutical agents such as neuropeptides across the  
blood-brain barrier and into brain tissue. The methods and  
pharmaceutical compositions thereof would desirably provide  
uniform introduction of the pharmaceutical agent throughout  
30 the brain and present as little risk to the patient as  
possible. Quite surprisingly, the present invention fulfills  
these and other related needs.

Summary of the Invention

Th present invention provides immunoliposomes and pharmaceutical compositions thereof capable of targeting pharmacological compounds to the brain. Liposomes are coupled to an antibody binding fragment such as Fab, F(ab')<sub>2</sub>, Fab' or a single antibody chain polypeptide which binds to a receptor molecule present on the vascular endothelial cells of the mammalian blood-brain barrier. Typically the antibody binding fragment is prepared from a monoclonal antibody. The receptor is preferably of the brain peptide transport system, such as the transferrin receptor, insulin receptor, IGF-I or IGF-2 receptor. The antibody binding fragment is preferably coupled by a covalent bond to the liposome.

Pharmacological compounds which especially benefit from this invention are those which are typically poorly transported across the blood-brain barrier, such as hydrophilic peptides, and which are often highly potent in small quantities in the brain. These include, for example, peptide neurotrophic factors, neurotransmitters and neuromodulators, e.g., beta-endorphins and enkephalins. For optimal delivery and to lessen the possibility of harmful side effects as a result of the liposome, the diameter of the liposome is less than 1 micron and typically smaller than about 0.45 microns.

In other aspects the invention provides methods for targeting a pharmacological compound to the blood-brain barrier of a mammal. A liposome containing the pharmacological compound is coupled to an antibody binding fragment which binds to a receptor molecule present on vascular endothelial cells of the mammalian blood-brain barriers, such as the transferrin receptor, insulin receptor, IGF-I or IGF-2 receptor. The pharmaceutical-containing immunoliposome is then administered to the subject in an amount sufficient to effectively treat or prevent the disorder. The preparation is usually administered parenterally, e.g., intravenously or intraarterially.



Description of the Specific Embodiments

The present invention provides compositions and methods for targeting drugs to and across the blood-brain barrier. The compositions, including pharmaceutical compositions, are comprised of a liposome, a pharmaceutical agent intended to be transported to the brain, and an antibody molecule or binding fragment thereof which binds to a receptor present on vascular endothelium cells of the brain.

Preferably the receptor is transferrin receptor and the antibody which binds to the receptor is a binding fragment which lacks some or all of the Fc portion of the molecule to minimize clearance of the composition by the reticuloendothelial system (RES).

Liposomes are used in the present invention to carry the pharmaceutical agent of interest to the blood-brain barrier for ultimate transport across the barrier and into the brain. As used herein and as recognized in the art, liposomes include any synthetic (i.e., not naturally occurring) structure composed of lipid bilayers that enclose a volume. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In the liposome preparations of the invention, the pharmaceutical agent to be delivered across the blood-brain barrier is incorporated as part of a liposome in conjunction with the targeting molecule.

The receptor to which the antibody fragment/liposome is directed may be one or several receptors present in vascular endothelium of the brain. The receptor should be sufficiently accessible to be bound by the antibody fragment coupled to the liposome. Receptors which are present in higher quantities in the brain capillaries than others are preferred for enhancing levels of transport across the blood-brain barrier. Receptors which may be targeted include, for example, transferrin receptor, insulin receptor, insulin-like growth factors I and II (IGF-I and IGF-II) receptors and other receptors of the brain peptide transport system, as generally described in Pardridge, Peptide Drug Delivery to the Brain,

Raven Press, New York, NY (1991), and U.S. Patent 4,801,575, which are incorporated herein by reference, the glucose transport receptor and the like.

5 Preferably the receptor targeted by antibody binding fragment is transferrin receptor. Transferrin receptor is selectively enriched on the endothelium of the brain microvascular endothelium of a variety of mammals, including humans, and is the primary pathway for iron to enter the brain. Iron-loaded transferrin, an 80 Kd glycoprotein, the  
10 principal iron transport protein in the circulation, undergoes transcytosis through the blood-brain barrier via the transferrin receptor. The structure and function of the transferrin receptor have been described in Seligman, Prog. Hematol. 13:131-147 (1983), which is incorporated by reference  
15 herein. Transferrin receptor is believed to project largely into the extracellular space. It can be isolated and purified from brain tissue using well known procedures. The purification, cloning, expression of human insulin receptor and the production of monoclonal antibodies thereto are  
20 described in U.S. Patent No. 4,761,371, which is incorporated herein by reference.

The targeted receptor purified from brain or expressed by recombinant DNA techniques may be used to produce antibodies, and preferably monoclonal antibodies, which may  
25 then be used to produce the antibody fragments useful in the present invention. The antibodies are made to the receptor molecule or a portion of the receptor molecule, such as that domain or domains which contributes to ligand binding. Preferably, the antibody will bind to an extracellular portion  
30 of the receptor which is proximate to the binding site of the receptor's native ligand, e.g., transferrin or insulin. In more preferred embodiments the antibody does not substantially block or compete with the binding of ligand to the receptor.

35 The production of monoclonal antibodies to transferrin receptor, including the OX-26 monoclonal antibody described in the Examples below, has been described in Jeffries et al., Immunology 54:333-341 (1985), which is incorporated herein by reference. Other monoclonal antibodies

to transferrin receptor have been described. See, e.g., the ATCC Catalogue of Cell Lines and Hybridomas, 7th ed., 1992, describing HB21, a murine IgG1 antibody which precipitates human transferrin receptor; HB84, a murine IgG2a monoclonal antibody to human transferrin receptor; and TIB219 and TIB220, rat anti-murine transferrin receptor antibodies. Monoclonal antibodies to the human insulin receptor have also been reported, including cell lines available from the ATCC, such as ATCC HB175 (J. Biol. Chem. 258:6561-6566 (1983)) and ATCC CRL 1827 (Curr. Top. Cell. Res. 27:83-94 (1985)), which are incorporated herein by reference.

Methods for the production of monoclonal antibodies are well known and may be accomplished by, for example, immunizing the animal with cells which express the receptor, substantially purified receptor, or fragments thereof as discussed above. Antibody producing cells obtained from immunized animals are immortalized and screened, or screened first for the production of antibody which binds to the receptor protein and then immortalized.

As an antibody fragment used in the present invention will generally lack the immunogenic Fc portion, the necessity of using human monoclonal antibodies is substantially avoided. However, it may be desirable to transfer antigen binding regions of the non-human antibodies, e.g., the hypervariable regions, to human framework regions by recombinant DNA techniques to produce substantially human Fab' molecules. Such methods are generally known in the art and are described in, for example, EPO publications 173,494 and 239,400, which are incorporated herein by reference. The production of single polypeptide chain binding molecules, also referred to as single chain antibodies, by recombinant DNA techniques is described in detail in U.S. Patent No. 4,946,778, which is incorporated herein by reference.

The antibody to the brain receptor molecule may be used intact or, more preferably, as a binding fragment thereof. By binding fragment is meant that the fragment retains an ability to specifically bind to an epitope of the target molecule of interest, such as transferrin or insulin

receptor, for example. Antibody binding fragments include at least the hypervariable or complementarity determining region (CDR) situated in an appropriate framework to produce a conformation which binds to the antigenic determinant. These binding fragments include, but are not limited to, Fv, Fab, F(ab')<sub>2</sub>, Fab', and single polypeptide chain binding molecules.

Antibody binding fragments can be produced from intact antibody molecules by a variety of procedures well known to those of skill in the art. For example, antibodies are usually fragmented by partial digestion with papain to produce two Fab fragments. Pepsin treatment can be used to produce the F(ab')<sub>2</sub> fragments where the two antigen binding domains are still bound together, i.e., as a multivalent binding molecule. Further reduction of the F(ab')<sub>2</sub> fragment with, e.g., dithiothreitol can be used to separate the antigen binding domains and produce two F(ab') fragments. The preparation of the various antibody fragments is described in detail in, e.g., Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1988 pp. 626-631, and Parham, in Cellular Immunology, 4th ed., (ed. D.M. Weir), vol. 1, chap. 14, Blackwell Scientific Publ., CA 1986, both of which are incorporated herein by reference. The polypeptide chains of the desired fragments may also be produced by a variety of recombinant DNA techniques and assembled intra- or extracellularly.

Once the desired antibody or, more preferably, an antibody binding fragment, has been obtained it is coupled to or incorporated into the liposome. Liposomes for use in the invention may be formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability in the bloodstream. Thus, the lipids which can be employed in the present invention include, e.g., cholesterol hemisuccinate and salts thereof, tocopherol hemisuccinate and salts thereof, a glycolipid, a phospholipid such as phosphatidylcholine,

ph sphatidylethanolamine (PE), phosphatidylserin ,  
phosphatidylglycerol, phosphatidic acid,  
phosphatidylinositol, sphingomyelin, and the like, alone or  
in combination. The phospholipids can be synthetic or derived  
5 from natural sources such as egg or soy. Phosphatidylcholines  
having a variety of acyl chain groups of varying chain length  
and degree of saturation are available or may be isolated or  
synthesized by well known techniques. In general, less  
saturated phosphatidylcholines are more easily sized,  
10 particularly when the liposomes are sized below about 0.3  
microns. The acyl chain compositions of phospholipid may also  
affect the stability of liposomes in the blood. Sterols such  
as cholesterol can be combined with the phospholipids. The  
phospholipids employed and the amount of sterol present  
15 depends on a number of factors such as lipophilicity of any  
added pharmaceutical agent, the targeting antibody fragment,  
and required properties of the liposome. These factors are  
generally known to those skilled in the art.

A wide variety of methods for preparing liposomes  
20 suitable for targeting to the blood-brain barrier in the  
present invention are available. See, for example, Szoka et  
al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), Liposome  
Technology, ed. G. Gregoriadis, CRC Press, Inc., Boca Raton,  
FL (1984), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028  
25 4,957,735 and 5,019,369, each of which is incorporated herein  
by reference. Since the targeted liposomes of the invention  
are not intended for uptake by the reticuloendothelial system,  
the lipid components are further selected to increase time in  
the bloodstream.

30 Following liposome preparation, the liposomes may be  
sized to achieve a desired size range and relatively narrow  
distribution of liposome sizes. For delivery to the brain,  
liposomes should generally be less than about 1.0 microns in  
siz , more pr ferably about 0.2 to 0.45 microns, which allows  
35 th lip some suspensi n to be steriliz d by filtrati n. F r  
sizing liposomes, a liposome suspension may be sonicated  
ith r by bath or prob down to small vesicles of less than  
about 0.05 microns in size. Homogenization may also be used,

which relies on shearing energy to fragment large liposomes into smaller ones. Homogenizers which may be conveniently used include microfluidizers produced by Microfluidics of Boston, MA. In a typical homogenization procedure, liposomes are recirculated through a standard emulsification homogenizer until selected liposome sizes, typically between 0.1 and 0.5 microns, are observed. In both methods the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

Extrusion of liposomes through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is an effective method for reducing liposome sizes to a relatively well defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller pore membranes to achieve a gradual reduction in liposome size.

Methods for coupling antibodies to liposomes generally involve either covalent crosslinking between a liposomal lipid and a native or modified antibody (or binding fragment thereof). In another approach, an antibody which has been covalently derivatized with a hydrophobic anchor, such as a fatty acid, is incorporated into a preformed lipid.

A variety of crosslinking agents can be employed to produce a covalent link between a lipid and the antibody. One protocol involves the derivatization of the free amino group of a PE with an amino reactive bifunctional crosslinker. The derivatized PE along with other lipids are then used to form liposomes by the methods described above. Once incorporated into the liposomes the derivatized PE can be reacted with the antibody by using the second reactive site on the crosslinking reagent. The use of heterobifunctional reagents are particularly useful because homocoupling between liposomes or between antibodies is avoided. Heterobifunctional crosslinkers which can be used include N-hydroxysuccinimidyl 3-(2-pyridylthio) propionate (SPDP), m-maleimidobenzyl-N-hydroxysuccinimide (MBS) and the like, and homobifunctional crosslinkers include toluene-2,4-diisocyanate (TDIC) and 1-

ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI)).  
Chemical crosslinking techniques also include the use of  
glutaraldehyde. These and other reagents are reviewed in  
Connor and Huang, Pharmac. Ther. 28:341-365 (1985), which is  
5 incorporated by reference herein.

The covalent attachment of antibody fragments to  
liposomes by crosslinking provides the advantage of attaching  
a large amount of antibody binding fragment to the liposome  
surface, with little or no loss of entrapped aqueous content  
10 using these methods. Care should be taken, however, that the  
crosslinking conditions are not so harsh that antibody binding  
is adversely affected, or that the liposomes and their  
contents are damaged. To avoid the orientation of antibody  
molecules which may render them incapable of binding to  
15 antigen, a spacer arm between the antibody and the lipid may  
optionally be employed.

A non-crosslinking association of antibodies or  
binding fragments thereof with liposomes can also be employed.  
This approach allows more than one type of antibody binding  
20 specificity per liposome, e.g., antibodies or fragments to  
different antigenic determinants on the transferrin molecule  
or even antibodies which bind to different receptors. The  
flexibility of the molecule minimizes steric hindrance which  
may block binding of the antibody to the antigen. Also, the  
25 reagents involved in the non-covalent methods are relatively  
mild and do not come into direct contact with the contents of  
the liposomes, thus avoiding possible damage to the liposome  
components.

In one method the non-covalent association of the  
30 antibody or fragment with the liposome can be accomplished by  
derivatizing the antibody component with a hydrophobic group.  
The derivatized antibody is then incorporated into the  
vesicles by inserting the hydrophobic anchor into the bilayer.  
Methods of derivatization include reacting the antibody with  
35 the N-hydroxysuccinimide ester of palmitic acid (NHSP) in the  
presence of 1-2% detergent, typically deoxycholate, as  
described in Huang et al., Biochim. Biophys. Acta 716:140-150

(1982). Alternatively, palmitic acid chloranhydride can replace NHSP as the acylation reagent.

Another method to acylate the antibody involves derivatizing the hydroxyl group of a PE or similar molecule rendering it capable of reacting with a sulfhydryl group. The antibody's disulfide bonds are then reduced with dithiothreitol (DTT) and then incubated with the derivatized PE. In yet another method useful in the present invention, a free PE or similar molecule is crosslinked to the carboxylic groups of an antibody by carbodiimide as described in Jansons and Mallett, Analyt. Biochem. 11:54-59 (1981), protecting the free amino groups of the antibody prior to the crosslinking to prevent homocoupling between antibody molecules.

Once derivatized, the antibody or binding fragment thereof can become associated into the liposomes by a variety of established protocols. For example, the derivatized antibody is mixed with the liposome in the presence of detergent, and the detergent then removed by dialysis to form the immunoliposomes. Another method involves a modification of the reverse-phase evaporation vesicle preparation method, where the preformed vesicles are mixed with antibody (lipid:antibody = 10:1 w/w) in a deoxycholate solution in the presence of ether, and both the detergent and ether are subsequently removed by extensive dialysis. See, e.g., Huang et al., in Methods. Enzymol. (1985). The preparation of immunoliposomes is also described in U.S. Patent No. 4,957,735, which is incorporated herein by reference. Preferably the antibody or binding fragment thereof is attached to the liposome component after the liposome is prepared.

A variety of drugs or other pharmacologically active agents are suitable for incorporation into the liposome and thus delivery to the blood-brain barrier. The use of liposomes as drug delivery vehicles is extensively reviewed in Gregoriadis, (ed.), Liposomes as Drug Carriers: Recent Trends and Progress, John Wiley & Sons, NY (1988), which is incorporated by reference herein. Therapeutic agents which may be delivered include protein neurotrophic factors, e.g.,



n rve growth factor, to treat brain injury and deg nerative  
dis as s, enzymes to replac those lost through genetic  
def cts causing metabolic storag diseas s, .g., Tay-Sachs  
disease, neurotransmitters and neuromodulators, e.g., dopamine  
5 and beta-endorphin for treating Parkinson's disease,  
conditions associated with pain, disorders of movement or  
cognition and behavior, antibiotics, chemotherapeutic agents,  
diagnostic imaging agents, etc. Particularly useful in the  
present invention are drugs such as peptides which have  
10 specific effects in the brain but which poorly cross into the  
brain normally, and have no or little effect in other organs.  
These compounds are often readily broken down in the  
bloodstream, and thus benefit from the protection provided by  
encapsulation in a liposome of the present invention. When  
15 targeted to a brain transport system as described herein, even  
a small amount crossing into the brain can provide a desired  
pharmacological effect.

One general class of drugs include water-soluble,  
liposome-permeable compounds which are characterized by a  
20 tendency to partition preferentially into the aqueous  
compartments of the liposome suspension, and to equilibrate,  
over time, between the inner liposomal spaces and outer bulk  
phase of the suspension. Representative drugs in this class  
include propranolol, ibuprofen, gentamicin, tobramycin,  
25 penicillin, theophylline, bleomycin, etoposide, n-acetyl  
cysteine, verapamil, vitamins, and radio-opaque and particle-  
emitter agents, such as chelated metals. Because of the  
tendency of these agents of equilibrate with the aqueous  
composition of the medium, it is preferred to store the  
30 liposome composition in lyophilized form, with rehydration  
shortly before administration. Alternatively, the composition  
may be prepared in concentrated form, and diluted shortly  
before administration.

A s cond g neral class of drugs are those which are  
35 water-solubl , but liposome-impermeabl . For the most part,  
these are peptide or prot in molecules, such as peptide  
hormon s, enzymes, enzyme inhibitors, apolip proteins, and  
higher molecular weight carbohydrates charact rized by long-

term stability of encapsulation. Representative compounds in this class include nerve growth factor, interferon ( $\alpha$ ,  $\beta$  and  $\gamma$ ), oxytocin, vasopressin, insulin, interleukins (e.g., IL-1, IL-2, etc.), superoxide dismutase, tumor necrosis factor, somatostatin, thyrotropin releasing hormone, and macrophage colony stimulating factor, among others. Peptide molecules and hormones which are typically very potent, and thus even small amounts crossing the blood-brain barrier can affect function, are particularly useful in targeted methods and compositions of the present invention. They include, for example, the  $\beta$ -endorphins and analogues, the enkephalins (relatively lipid soluble) such as Leu- and Met-enkephalins and analogues, melanocyte-stimulating hormone and melanocyte-stimulating hormone inhibitory factor,  $\beta$ -casomorphin, glucagon, delta sleep-inducing peptide and others, some of which are discussed in Banks and Kastin, supra, incorporated by reference herein.

A third class of drugs are lipophilic molecules which tend to partition into the lipid bilayer phase of the liposome, and which are therefore associated with the liposomes predominantly in a membrane-entrapped form. The drugs in this class are defined by an oil/water partition coefficient, as measured in a standard oil/water mixture such as octanol/water, of greater than 1 and preferably greater than about 5. Representative drugs include prostaglandins, amphotericin B, methotrexate, cis-platin and derivatives, vincristine, vinblastine, progesterone, testosterone, estradiol, doxorubicin, epirubicin, beclomethasone and esters, vitamin E, cortisone, dexamethasone and esters, betamethasone valerate and other steroids, etc.

Following treatment to remove free drug and/or targeting antibody molecule, the liposome suspension is brought to a desired concentration in a pharmaceutically acceptable carrier for administration to the patient. The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, but preferably they are administered parenterally, e.g., intravenously, intraarterially or intramuscularly. Thus, this invention

provides compositions for parenteral administration targeted for the blood-brain barrier which comprise a solution of a selected pharmaceutical contained in liposomes associated with an antibody molecule or binding fragment thereof which binds to a brain receptor molecule, preferably the transferrin receptor molecule, dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. These compositions may be sterilized techniques referred to above or produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The concentration of the liposomes in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing parenterally administrable liposomes formulations will be known or apparent to those skilled in the art and are described in detail in, for example, Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference, and Gregoriadis (1988), supra.

The dose and the route of administration and the carrier used may vary based on the disease being treated and in view of known treatments for such diseases. Amounts effective for a particular use will depend on the severity of the disease and the weight and general state of the patient

being treated. It must be kept in mind that the materials of the present invention may be employed in serious diseases, that is, life-threatening or potentially life threatening situations. In such cases, in view of the ability of the liposomes to target the blood-brain barrier and the minimal immunogenicity of the antibody binding fragments, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions.

The following examples are provided by way of illustration, not limitation.

#### EXAMPLE I

##### Production of Anti-Transferrin Receptor Fab'-Targeted Immunoliposomes

This Example describes the production of Fab'-coupled immunoliposomes for targeting to the transferrin receptor.

##### A. Preparation of Liposomes

Liposomes were prepared with a Heat Systems W 380 cup horn sonicator (Farmington, NY) by a method modified from Barrow and Lentz, Biochim. Biophys. Acta, 597:92-99 (1980). L- $\alpha$ -Phosphatidylcholine (PC) and N-[4-p-malimido phenyl butyryl] dioleoyl phosphatidylethanolamine (MPB-PE) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL), and cholesterol (Chol) was purchased from Sigma Chemical Co. (St. Louis, MO). Aliquots of chloroform (or chloroform/methanol) solution containing the appropriate lipids (PC/Chol/MPB-PE lipids at a molar ratio of 10/5/1), were pipetted into 5-ml glass test tubes. [ $^{14}$ C]-labeled dipalmitoylphosphatidylcholine (DPPC) (purchased from Amersham, Arlington Heights, IL), was added to give a final aqueous concentration of 0.5  $\mu$ Ci/ml. Solvents were evaporated under nitrogen, and solvent traces were removed under vacuum.

Lipid films were dispersed in 0.9% (w/v) saline (or H<sub>2</sub>O buffered 0.9% saline), transferred to 10 ml polycarbonate test tubes in 1-2 ml aliquots, and sonicated continuously at 50°C for 2-3 min to visual clarity. Liposomes were checked for diameter size by filtration through 0.45 micron-diameter cellulose acetate filters (Millipore, Boston, MA).

#### B. Preparation of Antibody Fragments

10 F(ab')<sub>2</sub> fragments were prepared from "OX-26" anti-transferrin receptor monoclonal antibody (purchased from Bioproducts for Science, Inc. (Indianapolis, IN) by Lofstrand Lab LTD (Gaithersburg, MD). Nonspecific rabbit IgG F(ab')<sub>2</sub> was purchased from Organon Teknika-Cappel (Durham, NC). Fab' fragments were generated from OX-26 F(ab')<sub>2</sub> (10 mg/ml) and nonspecific rabbit F(ab')<sub>2</sub> fragments (10 mg/ml) by reduction with 20 mM DTT (Sigma) in 20 mM HEPES (pH 6.5) for 60 min. Fab' fragments were separated from excess DTT on minicolumns of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), made from the barrels of 3-ml plastic syringes according to the technique of Fry et al., Anal. Biochem. 90:809-815 (1987). One-half ml aliquots of reduced Fab' were placed on previously centrifuged minicolumns and centrifuged at 1000 rpm for 10 min. The degree of reduction was determined by the method of Ellman, Arch. Biochem. Biophys. 82:70-77 (1959).

#### C. Coupling of Fab' Fragments to Liposomes

Freshly reduced Fab' fragments were added to equivalent volumes of freshly prepared liposomes (in 20 mM Hepes-buffered saline, pH 6.5) and mechanically stirred at room temperature overnight.

Fab'-coupled liposomes were isolated from the reaction mixture by flotation on a metrizamide (Sigma) gradient by the method of Heath et al., Biochim. Biophys. Acta 640:66-81 (1981). Briefly, 1 ml of liposomal suspension was mixed with 1 ml of 1 M metrizamide solution in 0.9% saline in a 5 ml nitrocellulose centrifuge tube. This solution was gently overlaid with 3 ml of 0.3 M metrizamide in 0.9% saline

and topped with 0.5 ml of 0.9% saline. The solution was centrifuged at 40,000 rpm for 1 h in a L8-80M ultracentrifuge (Beckman Instruments, Palo Alto, CA). The liposomes were pipetted off the gradient, and the m-trizamide was removed on a Sephadex G-25 minicolumn as previously described.

The protein concentrations of OX-26 anti-transferrin IgG Fab', and of nonspecific rabbit IgG Fab' (control) associated with the related liposomal formulations were estimated colorimetrically (Sigma Protein Assay Kit) to be 60 and 140 µg/ml, respectively.

Fab'-coupled liposomal formulations were not tested for receptor site binding. However, conjugates prepared from SATA-modified proteins have been shown to retain enzyme activity or function (Duncan et al., Anal. Biochem. 132:68-73 (1983)).

## EXAMPLE II

### Localization in the Brain of Immunoliposomes Targeted to Transferrin Receptor

This Example demonstrates that liposomes covalently coupled to Fab' fragments can bind to transferrin receptors in brain capillaries. Rats were used in these studies to demonstrate localization of the antibody fragment-targeted liposomes. Rats have a brain capillary system that, although smaller overall than the capillary system found in a human brain, is not substantially different. Further, radiolabeled OX-26 monoclonal antibody has been shown to bind isolated brain capillaries from rats and humans to approximately the same extent. Pardridge et al., J. Pharmacol. Exp. Ther. 259:66-70 (1991).

#### A. Intracarotid Infusion in Rats

Three-month-old male Sprague-Dawley rats (Charles River Breeding Laboratories, Lakeview, NJ) weighing approximately 200-300 g were given free access to food and

water and acclimated for at least 2 days to the Laboratory of Neurosciences animal room, where temperature, humidity and light cycles (6-18 h) were controlled. Experiments were conducted consistent with NIH guidelines, under a protocol approved by the NICHD Animal Care and Use Committee.

Intraarterial liposomal administration was undertaken to maximize liposome delivery and binding to the ipsilateral cerebral vasculature. The advantage of this administration route occurs only during the initial passage of liposomes through ipsilateral brain. Liposomes then enter the systemic circulation and show pharmacokinetic behavior similar to liposomes administered intravenously (Greig, N. in Implications of the Blood-Brain Barrier and its Manipulation, vol. 1: "Basic Science Aspects," (Neuwelt, E., ed.), pp. 311-368, Plenum Press, New York, NY (1989), which is incorporated herein by reference).

Rats anesthetized by i.p. injection of 50 mg/kg Na-pentobarbital (Ayerst, New York, NY) had sodium heparin-filled (100 I.U. in isotonic saline) catheters (size PE-50) inserted into the right external carotid and femoral arteries, according to the procedure of Rapoport et al., Am. J. Physiol. 238:R421-R431 (1980).

Liposomal formulations containing trace levels (0.5  $\mu\text{Ci/ml}$ ) of  $^{14}\text{C}$ -labeled DPPC were administered by infusion into the carotid artery catheter at a rate of 0.2 ml/min for 5 min (infusion pump No. 944, Harvard Instruments, Millis, MA). The carotid bifurcation was observed to ensure that the infusate passed cephalad into the internal carotid artery and not in a retrograde fashion down the common carotid artery. By linking the carotid catheter to a pressure transducer (Statham Instruments, Oxnard, CA), carotid artery pressure was monitored to ensure that hypertensive blood-brain barrier opening did not occur. After 1 or 10 min, the rats were killed by decapitation, and brain, kidneys, liver, and spleen were removed from each animal and were weighed. Plasma, brain (cerebrum ipsilateral and contralateral to infusion site, and cerebellum), other organ samples, and aliquots of liposomal formulations were measured for radioactivity (Tri-Carb 2200 CA

Liquid Scintillation Analyzer, Packard Instruments, Downers Grove, IL) according to the technique of Grig et al., J. Pharmacol. Exp. Ther. 245:1-7 (1988), which is incorporated herein by reference.

5

## B. Calculations

Radioactivity in organ samples was calculated as mean DPM/total DPM injected. Volumes of distribution ( $V_d$ ) for  $[^{14}\text{C}]$ -DPPC-labeled liposomal formulations were calculated as follows:  $V_d$ -DPM per gram wet tissue weight/DPM per ml plasma x 100 (% wet wt). In the calculation of radioactivity distributions, blood volume was assumed to be 6.5% of the total body weight (Altman, Blood and Other Body Fluids, Biological Handbook Series, Fed. Amer. Soc. Exp. Biol., Wash., D.C. (1961)). Tissue counts were corrected for counts present in residual blood (0.01 in brain, 0.1 in spleen, kidneys, and liver (ibid.)).

Assuming the liposomal diameter to be approximately 1000 Å, the lipid bilayer thickness 37 Å (Mason and Huang, Ann. N.Y. Acad. Sci. 308:29-48 (1978)), and the lipid (liposomal) density 0.9 g/cm<sup>3</sup>, the number of liposomes bound to the blood-brain barrier and the surface area occupied by bound liposomes were estimated. Capillary surface area in rat brain was estimated as 135 cm<sup>2</sup>/g wet weight brain (Altman, supra).

Dunnett's test was carried out to compare two means (Miller, R. Simultaneous Statistical Inferences pp. 67-87, McGraw-Hill, New York, Ny (1966)). A paired t-test was used to compare the perfused with unperfused sides of the brain of each rat. The value for statistical significance in all tests was  $P < 0.05$ .

The organ distributions of  $[^{14}\text{C}]$ -DPPC-labeled liposomal formulations following right-sided carotid infusion in 10 rats for each study are summarized in Table I. OX-26 liposomes showed a 50% greater association with brain than the corresponding non-specific Fab' controls at 1 and 10 min. Levels of radioactivity in brain of rats infused with OX-26 liposomes were statistically greater than in controls at 1 min



( $P < 0.05$ , Dunnett's test) but not at 10 min. Although brain levels were essentially unchanged at 10 min, statistical significance was not achieved because of greater variability associated with the data at 10 min.

5           Left-right differences in cerebrum distribution values in individual animals were assessed by paired t-test. Although values for cerebrum ipsilateral to infusion (right) were greater than those of contralateral (left) cerebrum, these did not reach statistical significance. OX-26 and OX-26  
10 control liposomes had similar liver uptakes; however, the spleen uptake of OX-26 liposomes was approximately 300% of controls at 1 and 10 min. OX-26 liposomes cleared from plasma more rapidly than controls. There was insignificant uptake of all liposomal formulations by kidney. Recoveries of  
15 all liposomal formulations averaged  $75 \pm 4\%$  (SEM) of the injected dose.

$V_d$  values, radioactivity per g wet weight of each organ divided by radioactivity per ml of plasma, normalize  
variability in plasma pharmacokinetics and liposomal plasma  
20 clearances among rats. The  $V_d$  values of the liposomal formulations (Table II) for OX-26 in brain were significantly greater than their respective controls at both 1 and 10 min, by approximately 2-fold at 1 min and by 6-8-fold at 10 min ( $p < 0.05$ , Dunnett's test). Although the  $V_d$  value for right  
25 cerebrum was up to 3.5 fold greater than left cerebrum, this did not reach statistical significance (paired t-test). At 1 and 10 min, high levels of OX-26 liposomes were present in liver and spleen, indicating liposomal accumulation in these organs.  $V_d$  values of all formulations in brain tissue did not  
30 increase between 1 and 10 min, indicating that liposomes did not measurably cross the blood-brain barrier.

          The foregoing results indicate that liposomes covalently coupled to antibody binding fragments, and particularly Fab' fragments can bind to transferrin receptors  
35 in brain capillaries. Bound liposomes were estimated to be approximately 0.03% of the injected dose or 0.025 pmol of liposomes. Control liposomes associated with rat brain were subtracted from total brain uptake of OX-26 liposomes because

brain radi activity levels are slightly elevated (above background) after intracarotid infusion (Micklus et al., Biochim. Biophys. Acta 1124:7-12 (1992)). Transferrin receptor density in rat brain has been estimated to be about 0.02 pmole per gram of brain (Pardridge et al., J. Pharmacol. Exp. Ther. 259:66-70 (1991)), generally equivalent to 0.034 pmole per brain for the rats used in the present experiments, or approximately 1 liposome per 1.3 receptors. OX-26 liposomal brain uptake remained constant rather than increasing after 1 min, suggesting that essentially all the receptor sites were occupied after 1 min. This result is consistent with the observation that OX-26 antibodies show significant binding to capillaries at 5 min after injection (Friden et al., Proc. Natl. Acad. Sci. USA 88:4771-4775 (1991)). No increased brain liposomal uptake was observed during the brief 10 min period used in this study.

Comparison of intra-animal left to right cerebrum distributions and  $V_d$  values of our liposomal preparations showed no statistical difference, indicating that receptor sites are rapidly saturated in both cerebral hemispheres. This suggests that similar receptor site saturation could be achieved by (1) intravenous administration of liposomes and/or (2) a reduction in the liposomal dose.

The capillary surface area in rat brain cortex is about 135 cm<sup>2</sup> per gram of brain (wet wt), or 230 cm<sup>2</sup> for rats in the present study; therefore, it was estimated that about 0.7% of brain capillary surface was covered with liposomes. Liposomal binding to brain can be increased to cover all of the capillary surface by upregulating the transferrin receptor or targeting other receptors, e.g., in a 270 g rat as used in this study, this would represent 3.8 pmol of liposomal brain uptake.

Transport of the Fab'-targeted liposome across the blood-brain barrier is demonstrated in experiments performed according to the brain capillary depletion procedure as described in Pardridge et al., J. Pharmacol. Exp. Therap. 259:66-70 (1991), and Triguero et al., J. Neurochem. 54:1882-1888 (1990), which are incorporated herein by reference.

In other aspects, OX-26 Fab'-coupled liposomes showed significant liver and spleen uptakes at 1 and 10 min after infusion. OX-26 liposomes demonstrated an unusually high spleen uptake, with 6% of their total radioactivity present in the spleen after 1 min, and 12% after 10 min. This enhanced uptake is likely due to the many transferrin receptors in spleen or on red blood cells, which are rapidly cleared by the spleen after binding to OX-26 liposomes. This suggests that OX-26 liposomes can be used to effectively target therapeutic agents to the spleen.

### EXAMPLE III

#### Liposomes Targeted to Insulin Receptor

This Example compares the ability of insulin-targeted liposomes to distribute labeled formulations in brain when compared to control preparations. Although the brain distribution of insulin-coupled liposomes was not statistically different from the control formulations,  $V_d$  values, which adjust data for plasma clearance, were higher for insulin liposomes in right cerebrum than controls at 1 and 10 min, suggesting that a moderate binding to brain capillaries does occur.

#### Formulations

The [ $^{14}\text{C}$ ]-DPPC-labeled liposomal formulations evaluated in this study consisted of 8  $\mu\text{mol/ml}$  suspensions of PC/Chol/MPB-PE lipids (molar ratio, 10/5/1), covalently coupled to proteins. The protein concentrations of insulin, of OX-26 anti-transferrin IgG Fab', and of nonspecific rabbit IgG Fab' (control) associated with the related liposomal formulations were estimated colorimetrically (Sigma Prot in Assay Kit) to be 250, 60 and 140  $\mu\text{g/ml}$ , respectively.

### A. Preparation of Thiolated Insulin

Insulin (porcin , sodium) and N-succinimidyl-S-acetyl-thioacetat (SATA) and w r obtain d from Cal Biochem Corp. (LaJ lla, CA). Insulin was thi lated with SATA  
5 by a modification of the method f Duncan et al., Anal. Biochem 132:68-73 (1983). Twenty  $\mu$ l of SATA in dimethyl formamide (DMF) (100 mg/ml) was reacted per 1 ml of insulin solution (10 mg/ml insulin + 5  $\mu$ l 5 N NaOH) in deionized water for 30 min. Thioacetylated insulin was deacetylated by  
10 reaction with 100  $\mu$ l (per ml insulin solution) of aqueous 0.5 M hydroxylamine (pH 6.8) for 30 min. Thiolated insulin was separated from low molecular weight solutes on minicolumns of Sephadex G-25, made from the barrels of 3-ml plastic syringes according to the technique of Fry et al., Anal. Biochem.  
15 90:809-815 (1978). One-half ml aliquots of thiolated insulin solution were placed on previously centrifuged minicolumns and centrifuged at 1,000 rpm for 10 min. After addition of 0.2 ml of saline, this centrifugation stop was repeated. The extent of thiolation was determined by the method of Ellman, supra.

20

### B. Coupling of Insulin to Liposomes

Freshly thiolated insulin was added to an equivalent volume of freshly prepared liposomes (in 20 mM Hepes-buffered saline, pH 7.5) and mechanically stirred at room temperature  
25 overnight. Insulin-coupled liposomes were isolated from the reaction mixture by flotation on a metrizamide gradient as described in Example I above.

The protein concentration of insulin associated with the liposomal formulation was estimated colorimetrically to be  
30 250  $\mu$ g/ml. Insulin-coupled liposomal formulations were not tested for receptor site binding. However, conjugates prepared from SATA-modified proteins have been shown to retain enzyme activity or function (Duncan et al., supra).

Animal studi s wer performed in accordance with the  
35 procedures described in Example II above.

The results, shown in Table I and Tabl II, indicate that insulin and insulin c ntrol lip som s had c mparable distributi ns in brain and, similarly, there were no

significant right-left cerebral differences following administration of either preparation. Insulin liposomes cleared from plasma more rapidly than control liposomes and had greater liver and spleen uptakes.

5            $V_d$  values of insulin liposomes in right cerebrum were significantly higher than control values at 1 and 10 min by approximately 2-fold ( $P < 0.05$ , Dunnett's test). Values in left cerebrum, however, were not different from controls, nor were there left-right differences in cerebrum  $V_d$  values.

10           The brain distribution of insulin-coupled liposomes was not statistically different from the control formulations, suggesting that they do not significantly bind to rat brain capillary. However,  $V_d$  values, which adjust data for plasma clearance, for insulin liposomes in right cerebrum  
15           (ipsilateral to liposome infusion) were higher than controls at 1 and 10 min, suggesting that a moderate binding to brain capillaries does occur. Possible reasons for such moderate binding are (1) low insulin binding affinity between liposome and capillary receptor (2) partial inactivation of the insulin  
20           during the liposomal coupling reaction, (3) low insulin receptor density at the cerebral capillary, and (4) competition between insulin liposomes with endogenous insulin for brain capillary receptor sites.

25

#### EXAMPLE IV

##### Liposomes Targeted Via Antibody Fragments to Insulin Receptor

30           A liposome is prepared according to Example I, incorporating a transportable neuropeptide to be targeted to the brain of an animal. A monoclonal antibody to human insulin receptor is digested to prepare Fab' fragments which are coupled to the liposome as described in Example I. The resulting targeting immunoliposome is combined with sterile  
35           saline to provide a physiologically acceptable solution, and is administered to the patient or subject parenterally.

## EXAMPLE V

Liposomes Targeted Via Antibody Fragments to IGF-I Receptor

5 A liposome is prepared according to Example I,  
incorporating a pharmacological compound to be targeted for  
delivery to the brain of a patient or subject. A monoclonal  
antibody to human IGF-I receptor is digested as described in  
Example I to prepare Fab' fragments. The Fab' fragments are  
coupled to the liposome as described in Example I. The  
10 resulting targeting immunoliposome is combined with sterile  
saline to provide a physiologically acceptable solution, and  
is administered to the patient or subject parenterally.

Table I

Distribution of [ $^{14}\text{C}$ ]-DPPC-labeled Liposomal Formulations in Rats:  
 \* DPM Recovered in Organs 1 and 10 Min after Intracarotid Injection.<sup>a</sup>

Min	Formulation	Brain	Cerebrum/ Right	Cerebrum/ Left	Cerebellum	Spleen	Liver	Plasma
1	OX-26 Fab'	.09±.01*	.05±.01*	.015±.002*	.03±.003*	6.1±.7*	14±1	34±4
	OX-26 Control	.06±.01	.02±.002	.01±.001	.02±.002*	1.9±.3	23±6	46±7
	Insulin	.05±.01	.03±.01	.01±.01	.01±.002*	2.4±.2*	31±4*	50±6*
	Insulin Control	.05±.002	.02±.003	.013±.002	.02±.002	0.8±.1	5±.6	73±3
10	OX-26 Fab'	.09±.02	.05±.01	.03±.01	.02±.004	11±2*	27±3	24±5
	OX-26 Control	.06±.01	.03±.01	.01±.002	.02±.004	3.0±.7	20±4	63±6
	Insulin	.05±.01	.03±.004	.015±.003	.01±.001*	3.3±.3*	34±4	45±5*
	Insulin Control	.06±.005	.02±.003	.02±.002	.02±.002	1.4±.1	10±1	67±5

<sup>a</sup> Data represent mean ± SEM (n = 10). OX-26 control is nonspecific rabbit IgG Fab'. Analysis of individual differences in right/left cerebrum values showed no significant difference, where P<0.05 (paired t test).

\* Denotes statistical difference from control value, where P<0.05 (Dunnnett's test).

Table II

$V_d$  Values for [ $^{14}\text{C}$ ]-DPPC-labeled Liposomal Formulations in Rats 1 and 10 Min after Intracarotid Injection.<sup>a</sup>

Min	Formulation	Cerebrum/Right	Cerebrum/Left	Cerebellum	Spleen	Liver
1	OX-26 Fab	2.6±.5*	0.7±0.1*	2.5±0.4*	220±50*	47±7
	Control Fab'	0.9±0.2	0.4±0.1	1.2±0.2	70±10	75±22
	Insulin	0.8±0.1*	0.5±0.1	0.9±0.1	74±12*	61±12
	Insulin Control	0.4±0.1	0.3±0.03	0.9±0.1	16±3	7±1
10	OX-26 Fab'	4.0±1.3*	2.5±1.0*	5.3±1.5*	1080±370*	200±60*
	Control Fab'	0.6±0.2	0.3±0.1	1.1±0.2	132±14	45±14
	Insulin	0.9±0.1*	0.5±0.1	1.0±0.1	130±20*	79±6*
	Insulin Control	0.5±0.05	0.4±0.05	1.0±0.1	28±3	16±2

<sup>a</sup> Data represent mean ± SEM (n = 10). OX-26 control is nonspecific rabbit IgG Fab'. Analysis of individual differences in right/left cerebrum values showed no significant difference, where  $P < 0.05$  (paired  $t$  test).

\* Denotes statistical difference from control value, where  $P < 0.05$  (Dunnnett's test).



It is evident from the above results that the subject invention provides antibody binding fragment-targeted liposomal formulations useful in the delivery of therapeutics and diagnostic compounds across the blood-brain barrier. In particular, the presence of antibody binding fragments in a liposome which are specific for brain transport receptor, such as transferrin receptor, insulin receptor, IGF-I or IGF-II receptor provides an effective means to enhance the uptake of certain drugs, such as hydrophilic drugs, peptides and proteins to the brain. The antibody binding fragment-modified liposomal formulations may be produced by a variety of means. Thus, increased efficacy, convenience and economics of lower dosages and less frequent administration to achieve therapeutic levels of drugs in the brain are among the advantages provided by compositions and methods of the present invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

## WHAT IS CLAIMED IS:

1. A composition which comprises a liposome coupled to an antibody binding fragment which binds to a receptor molecule present on vascular endothelial cells of a mammalian blood-brain barrier.

2. The composition of claim 1, wherein the antibody binding fragment is Fab, F(ab')<sub>2</sub>, Fab' or a single antibody chain polypeptide.

3. The composition according to claim 1, wherein the antibody fragment binds to a receptor of the brain peptide transport system.

4. The composition according to claim 3, wherein the receptor is transferrin receptor, insulin receptor, IGF-I or IGF-2 receptor.

5. The composition according to claim 1, wherein the antibody binding fragment is coupled by a covalent bond to the liposome.

6. The composition according to claim 1, wherein the liposome contains a pharmaceutical compound.

7. The composition of claim 6, wherein the pharmacologic compound is a hydrophilic neuropeptide.

8. The composition according to claim 6, further comprising a physiologically acceptable carrier.

9. The composition according to claim 1, wherein the diameter of the liposome is less than 1 micron.

10. The composition according to claim 9, wherein the diameter of the liposome is smaller than about 0.45 microns.

11. The composition of claim 1, wherein the antibody binding fragment is Fab, F(ab')<sub>2</sub> or Fab' which binds specifically to transferrin receptor.

12. The composition of claim 1, wherein the antibody binding fragment is prepared from a monoclonal antibody.

13. A pharmaceutical composition which comprises a antibody binding fragment which binds to a receptor molecule present on vascular endothelial cells of a mammalian blood-brain barrier, wherein the antibody binding fragment is coupled to a liposome which contains a pharmaceutical compound intended for treatment of a brain-associated disorder, and a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13, wherein the antibody binding fragment is Fab, F(ab')<sub>2</sub>, Fab' or a single antibody chain polypeptide.

15. The pharmaceutical composition of claim 14, wherein the receptor is transferrin receptor, insulin receptor, IGF-I or IGF-2 receptor.

16. A method for targeting a pharmacological compound for delivery to the blood-brain barrier of a mammal, which comprises:

administering to the mammal in need of the pharmacological compound a composition which comprises a liposome containing the pharmacological compound coupled to an antibody binding fragment which binds to a receptor molecule present on vascular endothelial cells of the mammalian blood-brain barrier.

17. The method of claim 16, wherein the composition is administered intravenously or intrarterially.

18. The method of claim 16, wherein the antibody binding fragment binds specifically to transferrin receptor, insulin receptor, IGF-I or IGF-2 receptor

5 19. The method of claim 11, wherein the antibody binding fragment is Fab, F(ab')<sub>2</sub>, Fab' or a single antibody chain polypeptide.

10 20. The method of claim 16, wherein the receptor is the transferrin receptor.

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO,A,91 04014 (SYNERGEN INC.) 4 April 1991 see page 1, paragraph 1 - page 2, paragraph 1 see page 4, paragraph 3 see page 5, paragraph 2 see page 5, paragraph 4 - page 6, line 1; claims	1-20
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 88 , June 1991 , WASHINGTON US pages 4771 - 4775 P. M. FRIDEN ET AL. 'ANTI-TRANSFERRIN RECEPTOR ANTIBODY AND ANTIBODY-DRUG CONJUGATES CROSS THE BLOOD-BRAIN BARRIER.' cited in the application see abstract	1-20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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BERTE, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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